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PRINCIPAL INVESTIGATOR: Kevin K. Caldwell, Ph.D.

CONTRACTING ORGANIZATION: University of New Mexico  
Albuquerque, New Mexico 87131-5041

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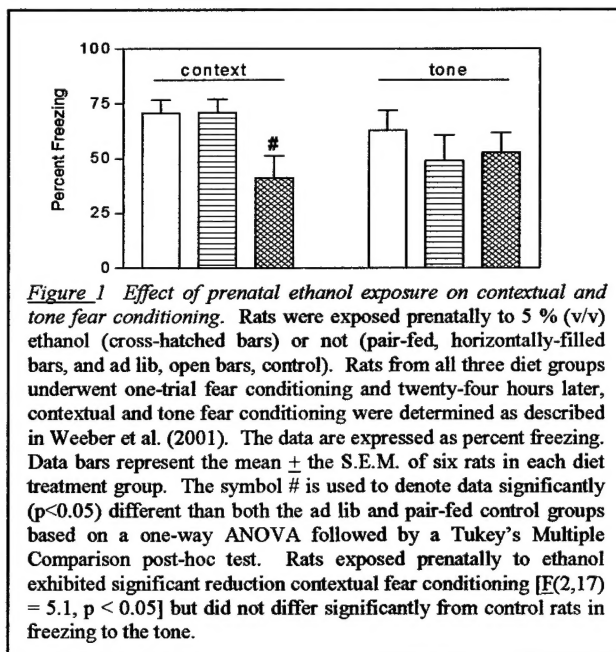
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## INTRODUCTION

Prenatal exposure to alcohol elicits a spectrum of neurochemical, neurobehavioral and neurophysiologic dysfunctions, depending on the amount and duration of maternal ethanol consumption. Heavy or binge patterns of drinking during pregnancy are associated with Fetal Alcohol Syndrome (FAS; Lemoine et al. 1968, Jones et al. 1973, Claren and Smith 1978), a group of profound morphological and neurological abnormalities observed in the offspring, whereas the consumption of moderate amounts of ethanol throughout pregnancy is associated with subtle cognitive and behavioral aberrations in offspring in the absence of the gross morphological deficits observed in FAS (Streissguth et al. 1990, Streissguth et al. 1994, Mattson and Riley 1998). Often, these cognitive and behavioral deficits become apparent only under stressful conditions (Streissguth et al. 1990). In 1996, the Institute of Medicine recommended an expansion of the classification of the effects of fetal alcohol exposure to include a new category termed Alcohol-Related Neurodevelopmental Disorders (ARND; Stratton et al. 1996). In order to develop effective strategies for the treatment of the learning and memory decrements in ARND, the underlying neurobiological mechanisms need to be elucidated. The overall aim of the funded project is to uncover biochemical mechanisms that underlie these learning and memory failures, using a rat model of moderate fetal alcohol exposure (FAE) and a behavioral technique termed fear conditioning.

In rats, prenatal exposure to alcohol has been associated with a variety of learning/memory



impairments, including spatial-navigation assessed using the Morris water maze task (Gianoulakis 1990, Westergren et al. 1996) and reduced retention of learned fear (Figure 1 from Weeber et al., 2001). One-trial fear conditioning, which we employed in the studies shown in Figures 1 and 2, is a form of classical conditioning in which a conditioned stimulus (CS), a tone, is paired with an unconditioned stimulus (UCS), a brief electrical foot shock. The animal learns to fear, or prepare for, the approach of the UCS in subsequent trials. In addition to learning to fear the explicit CS, animals learn the association between the shock experience and the constellation of environmental cues that form the training context. Fear responses to the CS and to the context are separable and can be measured as the duration of conditioned "freezing"

(Bouton & Bolles, 1980; Fanselow, 1980).

In recently published studies (Weeber et al., 2001), we reported that one-trial fear conditioning is associated with time-dependent changes in subcellular phospholipase C- $\beta$ 1a (PLC- $\beta$ 1a) enzyme activity and protein level in the hippocampal formation and that these changes are altered in rats that were exposed prenatally to ethanol. PLC- $\beta$ 1a is one of at least 11 distinct mammalian PLC isozymes that have been identified (Rhee 2001). In general, PLC- $\beta$  isozymes are regulated by G-protein-coupled receptors and PLC- $\gamma$  isozymes are regulated by tyrosine kinase receptors. Each of the 11 isozymes hydrolyze phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) to yield the intracellular second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>, IP<sub>3</sub>) and 1,2-

diacylglycerol (DAG) (Majerus et al. 1990; Williams 1999). Ins(1,4,5)P<sub>3</sub> releases Ca<sup>2+</sup> from intracellular storage sites; DAG directly activates certain forms of protein kinase C (PKC) (Berridge 1993; Nishizuka 2001). Changes in the cellular levels and turnover of PtdIns(4,5)P<sub>2</sub>, Ins(1,4,5)P<sub>3</sub> and DAG play important roles in various cellular processes including secretion, growth, and neuronal plasticity (Berridge 1993; Nishizuka 2001).

## **BODY**

Two goals were listed in the approved Statement of Work.

**GOAL 1:** To determine the time course of one trial fear conditioning-induced changes in PLC-β1a subcellular distribution and catalytic activity in adult rats who were exposed, or not, to moderate levels of ethanol throughout gestation.

**Progress made toward GOAL 1 is as follows.** We have chosen to postpone work on this specific aim in order to direct all of our efforts toward GOAL 2. The tissue that was collected during year one of the grant (see annual report for 2001 for detail) remains available to us.

**GOAL 2:** To determine whether one trial fear conditioning alters the phosphorylation state of PLC-β1a and, if so, whether fetal alcohol exposure modifies the changes in PLC-β1a phosphorylation.

**Progress made toward GOAL 2 is as follows.**

### **A.) Characterization of the interaction between PLC-β1a and PKC-α.**

In our 2001 annual report, we demonstrated that rat brain PLC-β1a forms a tight complex with protein kinase C-α (PKC-α) and concluded that PKC-α could be responsible for the down-regulation of PLC-β1a enzyme activity that we have observed following fear conditioning (Weeber et al. 2001). The significance of the observed interaction between PLC-β1a and PKC-α is indicated by reports demonstrating that PKC isozymes play an important role in learning and memory (Weeber et al. 2000; Colombo et al. 1997; Fordyce et al. 1995).

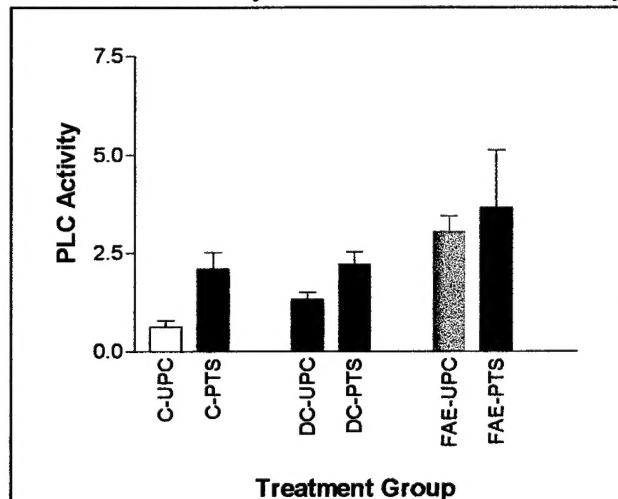
We have performed preliminary studies aimed at determining the molecular basis of the interaction between PLC-β1a and PKC-α and concluded that the association is indirect. That is, PLC-β1a and PKC-α do not directly bind to each other; instead, both bound by an intermediary (e.g., scaffolding) protein. This conclusion is based on the following information. Recently, Suh et al. (2001) reported that PLC-β isozymes, including PLC-β1a, possess carboxyl-terminal motifs that bind regions of proteins known as PDZ-domains. PDZ domains, which were originally identified as conserved sequences in PSD-95/SAP90, DLG, and ZO-1 proteins, are peptide motifs of approximately 100 amino acids that bind to proteins whose carboxyl-terminal amino acid sequence fits the consensus sequence, X-(S/T)-X-(V/L), where X is any amino acid and S, T, V and L designate serine, threonine, valine and leucine, respectively (Suh et al. 2001). The carboxyl-terminal of PLC-β1a, D-T-P-L, fits this consensus sequence. While PKC-α has not been reported to possess a PDZ-domain, its C-terminal, Q-S-A-V, does fit the consensus sequence for a PDZ domain binding motif. Thus, both PLC-β1a and PKC-α are capable of binding PDZ domain-containing proteins, and thus such a protein could act to bring PLC-β1a and PKC-α into a complex. A candidate for this protein is a member of the family of Na<sup>+</sup>/H<sup>+</sup>-exchanger regulatory factors, which contain two PDZ domains and which have been shown to bind PLC-β1a (Tang et al. 2000). In support of our conclusion that the C-terminal PDZ domain binding motif of PKC-α is important for the association with PLC-β1a, we have found that, when we immunoprecipitated PKC-α employing an antibody that was raised against the C-terminal amino acid sequence of the protein (Transduction Labs, Lexington, KY), PLC-β1a was not

present in the immunoprecipitate (data not shown), yet PLC- $\beta$ 1a was associated with PKC- $\alpha$  when an anti-PKC- $\alpha$  antibody raised into an internal epitope was employed (Figure 2 of 2001 annual report).

### B.) Studies demonstrating an interaction between PLC isozymes and extracellular signal-regulated protein kinases

The majority of our work during the last year has been aimed at studying the association of PLC isozymes with extracellular signal-regulated protein kinase 2 (ERK2). ERK2 is a mitogen-activated protein kinase (MAPK). MAPKs are proline-directed serine/threonine kinases; that is, they phosphorylate serine and threonine residues adjacent and N-terminal to proline. ERK2 has been implicated in several learning paradigms, including contextual fear conditioning (Atkins et al., 1998; Selcher et al. 1999; Schafe et al. 2000), novel taste (Berman et al., 1998; Swank and Sweatt 2001), inhibitory avoidance (Walz et al. 2000), and Morris water maze (Blum et al., 1999; Selcher et al. 1999). In addition, ERK2 has been implicated in long-term potentiation (LTP) and long-term depression (LTD) models of learning. It follows that cellular components (e.g., PLC isozymes) that interact either directly or indirectly with ERK2 are important to the processes underlying these forms of learning and memory.

Our first line of study was to determine if PLC enzyme activity was associated with ERK2 in rat



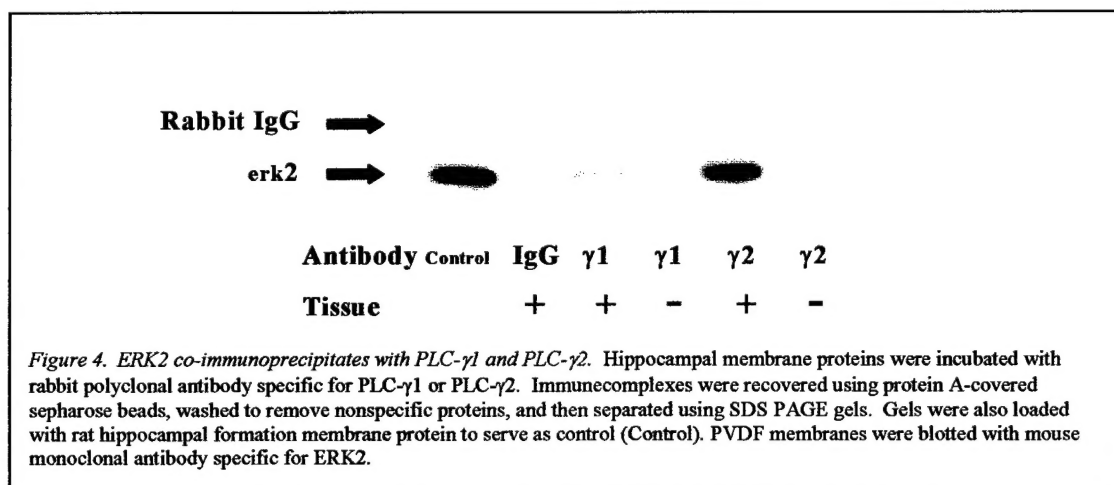
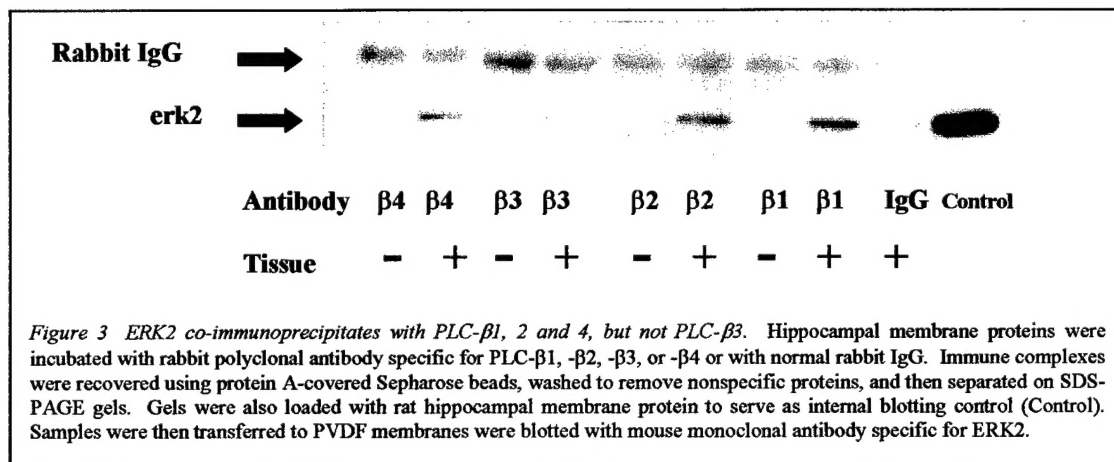
**Figure 2** PLC activity present in anti-ERK2 immunoprecipitates isolated from rat hippocampal formation postnuclear fractions. The hippocampal formation was isolated from adult rats that had been prenatally exposed to ethanol (fetal alcohol exposure, FAE), or were the offspring of dams receiving an ethanol-free liquid diet (diet control, DC) or had ad lib access to rat chow (control, C). Following fear conditioning (paired tone-shock, PTS) or behavioral control (unpaired control, UPC), the hippocampal formation was isolated. Postnuclear fractions were prepared and ERK2 was immunoprecipitated. Immune-complex PLC activity was measured and expressed as nmole Ins(1,4,5)P<sub>3</sub> product formed / min / µg tissue. Two-way ANOVA revealed a significant effect of diet [ $F(2,21)=5.35$ ,  $p<0.02$ ] but no significant effect of fear conditioning, nor a significant interaction between diet and fear conditioning.

brain. In addition, we sought to determine whether fear conditioning and/or FAE exerted an effect on the amount of PLC enzyme activity associated with ERK2. For these studies, anti-ERK2 immunocomplexes were isolated from the hippocampal formation of rats that had either undergone one-trial fear conditioning (paired tone-shock, PTS) or an "unpaired" control (UPC) paradigm and had been exposed (fetal alcohol exposure, FAE) or not (ad lib control, C, and liquid diet control, DC) prenatally to alcohol. The amount of PLC catalytic activity associated with the immunocomplexes was assessed by measuring the *in vitro* hydrolysis of PtdIns(4,5)P<sub>2</sub>. Figure 2 demonstrates that anti-ERK2 immunoprecipitates do, indeed, contain measurable amounts of PLC enzyme activity and that the amount of PLC activity associated with these immunoprecipitates is increased following fear conditioning of control (C and DC) rats. In contrast, PLC enzyme activity in FAE rat hippocampal formation was elevated in behavioral control (UPC) animals and does not change with fear conditioning (compare FAE-UPC and FAE-PTS). These results indicate that

FAE alters the interaction of one or more PLC isozymes with ERK2, and, thereby, alters the effect of behavioral challenge on this association. It is our hypothesis that an increase in the

amount of PLC activity associated with ERK2 is responsible for the generation of a signal (i.e., change in intracellular PtdIns(4,5)P<sub>2</sub>, Ins(1,4,5)P<sub>3</sub>, and DAG levels) that is important for conditioned-fear learning and that the lack of this signal in FAE rats underlies, at least in part, the reduced contextual fear learning observed in these animals (see Figure 1).

We next sought to determine which PLC- $\beta$  and/or PLC- $\gamma$  isozymes associate with ERK2 in rat hippocampal formation. Anti-PLC- $\beta$  and anti-PLC- $\gamma$  immunocomplexes were isolated from hippocampal formation membrane extracts and probed for anti-ERK2 immunoreactivity. We found that ERK2 co-immunoprecipitated with PLC- $\beta$  1a, - $\beta$ 2, - $\beta$ 4, - $\gamma$ 1 and - $\gamma$ 2 (see Figures 3 and 4). In contrast, the amount of anti-ERK2 immunoreactivity associated with anti-PLC- $\beta$ 3 immunoprecipitates was not noticeably different than background (i.e., IgG control). Of the PLCs that co-immunoprecipitate ERK2, we have reproducibly found that anti-ERK2 immunoreactivity signals were most robust for PLC- $\beta$ 2, PLC- $\gamma$ 1, PLC- $\gamma$ 2. When anti-PLC- $\beta$  and PLC- $\gamma$  immunoprecipitates were probed for anti-ERK1, anti-phospho-ERK1 and anti-phospho-ERK2 immunoreactivities, no, or minimal, signals were detected (data not shown).



The demonstration that PLC- $\beta$ 1, - $\beta$ 2, - $\beta$ 4, - $\gamma$ 1, and - $\gamma$ 2 interact with ERK2, which plays an important role in fear-conditioned learning (see above), indicates that these PLC isozymes also play important roles in the biochemical processes underlying learning and memory formation.



We have begun to investigate the physiologic effect of these interactions. In these studies we have found that incubation of anti-PLC- $\beta$ 1a immunoprecipitates with (catalytically active)

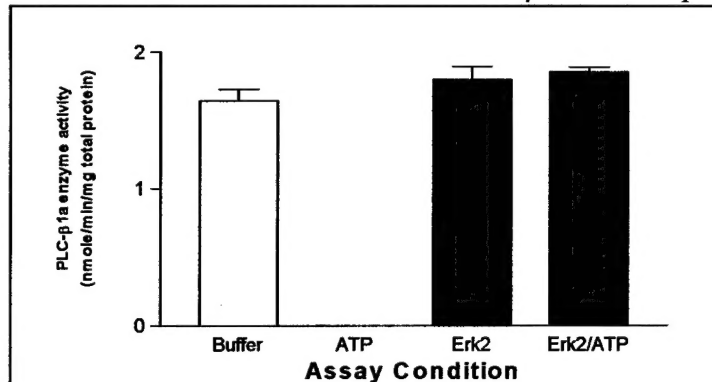


Figure 5. Lack of an effect of phospho-ERK2 treatment of anti-PLC- $\beta$ 1a immunoprecipitates on associated PLC activity. PLC- $\beta$ 1a, immunoprecipitated from rat hippocampal formation postnuclear fractions, was incubated in presence or absence of activated (phospho-) ERK2 and the presence or absence of ATP, according to the manufacturer's directions (Upstate Biotechnology, Inc., Lake Placid, NY). Immunoprecipitates were subsequently washed three-times with PLC assay buffer and PLC activity was measured as described in Weeber et al. (2001). A one-way ANOVA revealed that there were no significant differences between the treatment groups [ $F(3,22)=3.12$ ].

phospho-ERK2 *in vitro* does not significantly alter PLC- $\beta$ 1a enzyme activity. These results are in agreement with Xu et al. (2001), who report that phosphorylation of PLC- $\beta$ 1 by ERK does not change enzyme activity. It is possible that, rather than regulating PLC- $\beta$ 1 catalytic activity, ERK-dependent phosphorylation of PLC- $\beta$ 1 controls interaction of the enzyme with other proteins.

In conclusion, we have made several important observations in these studies. First, we identified that ERK2 associates with PLC- $\beta$ 1, - $\beta$ 2, - $\beta$ 4, - $\gamma$ 1 and - $\gamma$ 2 isozymes. Other than the report of Xu et al. (2001) demonstrating that ERK2 associates with PLC-

$\beta$ 1, these are novel observations. Further, the studies of Xu et al. (2001) employed transformed fibroblasts (3T3 cells) overexpressing PLC- $\beta$ 1, whereas our studies have employed normal rat brain. Second, we demonstrate that the association is not specific for PLC- $\beta$ 1, but also occurs with PLC- $\beta$ 2, - $\beta$ 4, - $\gamma$ 1 and - $\gamma$ 2. It is worth noting that the relative levels of these associations are not directly related to the relative levels of these PLC isozymes in the hippocampus (our unpublished data), indicating that there are differences in either the affinity of the interactions or differences in compartmentalization of ERK2 and the individual PLC isozymes. Interestingly, we did not detect an association between PLC- $\beta$ 3 and ERK2. It is possible that this evidence will provide a clue about the mechanism of association between ERK2 and PLC isozymes. We are currently assessing this possibility. Third, in contrast to the work of Xu et al. (2001), we did not detect an association between PLC- $\beta$ 1 and phospho-ERK1/2. Xu et al. (2001) used an antibody that recognizes both PLC- $\beta$ 1a and PLC- $\beta$ 1b and, thus, it is possible that phospho-ERK1/2 associates with PLC- $\beta$ 1b but not with PLC- $\beta$ 1a. Similarly, we found that PLC- $\beta$ 2, - $\beta$ 3, - $\beta$ 4, - $\gamma$ 1 and - $\gamma$ 2 isolated from hippocampal membranes do not appear to be complexed with phospho-ERK1/2. Fourth, we were unable to detect an association between any of the PLC- $\beta$  or PLC- $\gamma$  isozymes and ERK1. Finally, the finding that there was increase in the amount of PLC activity associated with anti-ERK2 immunoprecipitates following fear-conditioned learning indicates that the interaction between ERK2 and PLC isozymes is of physiologic significance and may play a role in FAE-induced learning deficits..

## KEY RESEARCH ACCOMPLISHMENTS

- We have made three important novel observations: First, we found that FAE increases the amount of PLC activity associated with ERK2 in the hippocampal formation isolated from control animals. Second, we found that FAE modifies the effect of fear conditioning on the



association between ERK2 and PLC. Third, we identified that ERK2 associates with PLC- $\beta$  and PLC- $\gamma$  isozymes in rat hippocampal formation.

- We have initiated studies on the effect of ERK2 phosphorylation on PLC enzyme activity.

## REPORTABLE OUTCOMES

1. Severns V., Savage D.D. and Caldwell K.K. (2002) Effects of prenatal ethanol exposure on phospholipase C- $\beta$ 1a enzyme of the hippocampal formation of mice. National Institute of Mental Health Career Opportunities in Research Education and Training (COR) Colloquim. April 10-14, 2002. Minneapolis, MN.
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## CONCLUSIONS

These studies have provided important information on the biochemical basis of FAE-induced learning and memory deficits. The observed effects of FAE on the association of ERK2 and PLCs have been published in abstract form (see Reportable Outcomes above) and are currently being prepared for submission as a full-length journal article. Further, we believe that the observed interactions between ERK2 and PLC- $\beta$  and PLC- $\gamma$  isozymes are important findings and, with additional study aimed at uncovering the molecular basis of the association, will be published.

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## FETAL ETHANOL EXPOSURE DISRUPTS SIGNAL INTEGRATION IN THE HIPPOCAMPAL FORMATION OF ADULT RAT OFFSPRING.

C.T. Buckley, D.D. Savage and K.K. Caldwell

University of New Mexico, Albuquerque, NM, 87131-5223.

Maternal consumption of moderate levels of ethanol causes subtle cognitive and behavioral aberrations in the offspring. We hypothesize that defects in the integration of cellular signaling are important components of the neurochemical imbalances that underlie these cognitive and behavioral abnormalities. Previously, we reported that rats that were exposed prenatally to ethanol displayed attenuated contextual fear conditioning and altered regulation of hippocampal formation phospholipase C (PLC) activity (Veeber et al. *Neurobiology of Learning and Memory* 76, 151-182, 2001). As contextual fear conditioning has been shown to be dependent upon hippocampal formation extracellular signal-regulated kinase 2 (ERK2) activity, we tested the hypothesis that the integration of hippocampal formation PLC- and ERK2-dependent signaling is perturbed in adult offspring who were exposed to ethanol prenatally. In diet control rats, we have found that hippocampal PLC activity co-immunoprecipitates with ERK2, and that 30 minutes following fear conditioning there is a significant increase in the amount of PLC activity present in anti-ERK2 immunocomplexes compared to complexes isolated from behavioral control rats. In contrast, anti-ERK2 immunocomplexes isolated from the hippocampal formation of rats exposed prenatally to ethanol do not show significant changes in associated PLC activity 30 minutes following fear conditioning. In related studies, we have found that 24 hours after fear conditioning, hippocampal PKC-b2 and PKC-e activities are decreased. We speculate that in fetal alcohol-exposed rats, behavioral challenge elicits spatially and temporally inappropriate PLC-, PKC- and ERK2-dependent signaling, which, in turn, disrupts downstream signaling cascades that are important for contextual learning and memory. This research was supported by National Institute on Alcohol Abuse and Alcoholism Grants AA12635 (KKC) and AA12400 (DDS) and U.S. Department of Army Award #DAMD17-00-1-0582 (KKC).

## ABSTRACT

Maternal consumption of moderate levels of ethanol causes subtle cognitive and behavioral alterations in the offspring. We hypothesize that defects in the integration of cellular signaling are important components of the neurochemical imbalances that underlie these cognitive and behavioral abnormalities. Previously, we reported that rats that were exposed prenatally to ethanol displayed attenuated contextual fear conditioning and altered regulation of hippocampal formation phospholipase C (PLC) activity (Weeber et al. 2001). As contextual fear conditioning has been shown to be dependent upon hippocampal formation intracellular signal-regulated kinase 2 (ERK2) activity, we tested the hypothesis that the integration of hippocampal formation PLC- and ERK2-dependent signaling is perturbed in adult offspring who were exposed to ethanol prenatally. In diet control rats, we have found that hippocampal PLC activity co-immunoprecipitates with ERK2, and that 30 minutes following fear conditioning there is a significant increase in the amount of PLC activity present in the anti-ERK2 immunoprecipitates compared to complexes isolated from behavioral control rats. In contrast, anti-ERK2 immunoprecipitates isolated from the hippocampal formation of rats exposed prenatally to ethanol do not show significant changes in associated PLC activity 30 minutes following fear conditioning. In related studies, we have found that 24 hours fear-conditioning, hippocampal PKC- $\beta$ 2 and PKC- $\alpha$  activities are decreased. We speculate that in fetal alcohol-exposed rats, behavioral challenge elicits spatially and temporally inappropriate PLC, PKC, and ERK2 dependent signaling, which, in turn, disrupts downstream signaling cascades that are important for contextual learning and memory.

## INTRODUCTION

Maternal consumption of moderate levels of ethanol causes subtle cognitive and behavioral alterations in the offspring (1). We hypothesize that defects in the integration of cellular signaling are important components of the neurochemical imbalances that underlie these cognitive and behavioral abnormalities. Recently, we found that rats who were exposed prenatally to ethanol displayed reduced retention of learned fear after one trial fear conditioning (2). In the proposed studies, we tested the hypothesis that the integration of hippocampal formation phosphatidylinositol-specific phospholipase C (PLC) and extracellular signal-regulated kinase (ERK2)-dependent signaling is perturbed in adult offspring who were exposed to ethanol prenatally. As a result, behavioral challenge elicits spatially and temporally inappropriate PLC- and ERK2-dependent signaling, leading to cognitive and behavioral impairments activities

## MATERIALS AND METHODS

**Fetal Alcohol exposure paradigm**  
All of the procedures involving animals were approved by the University of New Mexico Laboratory Animal Care and Use Committee. Prenatal exposure of rats to ethanol was performed as described previously (2). Additionally, fear conditioning of rats was performed as described (2).

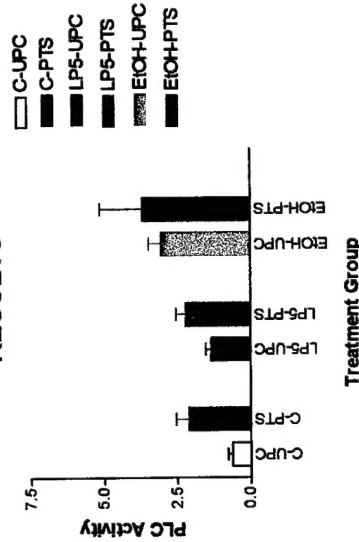
### Tissue Preparation and Subcellular Fractionation Procedures

Female Sprague Dawley rats received fear-conditioned training as described by Weeber et al. 2001. Thirty minutes post training, rats were killed by decapitation and their hippocampus was bilaterally removed, suspended in ice-cold homogenization buffer (HB), and homogenized in Wheaton homogenizers 5 strokes with the loose and 5 strokes with the tight pestal. Rat hippocampal tissue homogenates were loaded into a Sorval RC-3B centrifuge, and spun at 1,000 x g for 7 minutes. The supernatant, the S1 fraction, was decanted from the pellet, the P1 fraction. The P1 suspension was resuspended and returned to the centrifuge and spun again at 7 minutes. The supernatant was decanted, combined with the supernatant from the first centrifugation to form the complete S1 fraction, and spun using an Oxygene TLX ultracentrifuge at 200,000 x g for thirty minutes. The soluble fraction, the S2 fraction, was decanted from the pellet, the P2 fraction. The P2 pellet was then homogenized in 500  $\mu$ l of HB containing 75 mM NaCl, 75 mM KCl, 1% Triton X-100 and as described above, left on ice for Triton X-100 extraction, and spun in the ultracentrifuge at 200,000 x g for thirty minutes. The Triton X-100 soluble P2 fraction was decanted and stored at -60°C.

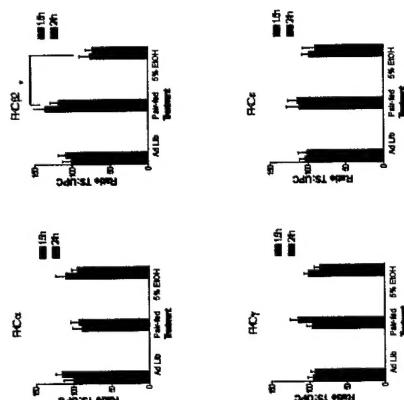
### Immunoprecipitation of ERK2

ERK2 was immunoprecipitated from the postnuclear subcellular fraction as described for the immunoprecipitation of PLC- $\beta$ 1a (2). PLC enzyme activities were measured as described (2).

## RESULTS



**Figure 1** PLC activity present in anti-ERK2 immunoprecipitates isolated from rat hippocampal formation postnuclear fractions. The hippocampal formation was isolated from adult rats that had been prenatally exposed to ethanol (5% ethanol diet, EIOH), or were the offspring of dams receiving an ethanol-free liquid diet (Pair-fed, LP5) or had no access to rat chow (control, C). Following fear conditioning (paired tone-shock, PTS) or behavioral control (unpaired control, UPC), the hippocampal formation was isolated. Postnuclear fractions were prepared and ERK2 was immunoprecipitated. Immunoprecipitated PLC activity was measured and expressed as nmoles hist(1,4,5)P<sub>3</sub> product formed / min / μg tissue.



**Figure 2** Membrane bound PKC isotypes in the hippocampus at 1.5 and 24 hr post-CFC. The membrane fraction of hippocampal homogenates was resuspended and electrophoresed on 10% polyacrylamide gels. Bands were probed with rabbit anti-PKC- $\alpha$ ,  $\beta$ 2,  $\gamma$ , and  $\epsilon$  primary antibodies and HRP-conjugated secondary antibodies and visualized by enhanced chemiluminescence. Band densities were analyzed using a two-tailed one-way ANOVA and Bonferroni's multiple comparison post-hoc test. The X-axis represents the three rat dam diets. The Y-axis represents the ratio of the post-hoc test. The appropriate PKC isotype. Tone shock (TS) represent animals that underwent contextual fear conditioning (CFC), while unpaired controls (UPC) represent control animals that experienced the same tone and shock in an unpaired fashion. (\* = p<0.05; error bars are  $\pm$  SEM).

## CONCLUSIONS

In these studies we found that the amount of PLC enzyme activity associated with ERK2 in the basal (i.e., UPC) state in fetal alcohol-exposed rat hippocampal formation is significantly higher than in diet control rats. Further, we did not observe a fear conditioning-dependent increase in the amount of PLC activity associated with anti-ERK2 immunoprecipitates in fetal alcohol rats, as was observed in diet control rats.

Recently, it has become appreciated that the components of signal transduction systems often are not isolated from each other, but, rather, are present in the cell as complexes. We have found that one or more PLC isozymes is associated with ERK2 in rat hippocampal formation. In addition, we found fetal alcohol exposure blocks the effects of fear-conditioned learning and memory on this association. Because ERK2 has been shown to be critical for hippocampal-dependent learning and memory, the impairing effects of fetal alcohol exposure may contribute to the learning deficits observed in animals exposed prenatally to ethanol.

Additionally, our data, to this date, represent a significant decrease in membrane bound PKC  $\beta$ 2 in FAE animals versus isochronic, pair-fed controls. Levels of PKC isotypes in the cytosol are currently being evaluated.

These studies indicate that the learning impairing effects of FAE may be due to damage to hippocampal signal transduction systems.

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## ACKNOWLEDGMENTS

This research was supported by National Institute on Alcohol Abuse and Alcoholism Grants AA12635 (KCC) and AA06548 (DDs) and U.S. Department of Army Awards #DAMD17-00-1-0582 (KCC).

**EFFECTS OF PRENATAL ETHANOL EXPOSURE ON PHOSPHOLIPASE C- $\beta$ 1A ENZYME OF THE HIPPOCAMPAL FORMATION IN MICE.** Virginia Severns, Department of Neuroscience, University of New Mexico School of Medicine, Albuquerque, NM.

*Faculty Mentors:* Kevin K. Caldwell, Department of Neuroscience, University of New Mexico School of Medicine, Albuquerque, NM; and Daniel D. Savage, Department of Pharmacology, University of New Mexico School of Medicine, Albuquerque, NM.

Maternal consumption of moderate levels of ethanol causes subtle cognitive and behavioral aberrations in offspring. In mice, prenatal exposure to alcohol has been associated with decrements in various measures of learning, including spatial and operant learning. We hypothesize that alternations in phosphatidylinositol-specific phospholipase C- $\beta$ 1a (PLC- $\beta$ 1a)-dependent signaling may underlie these learning deficits. Phosphorylation has been shown to influence enzyme catalytic activity of a variety of enzymes, including PLC- $\beta$ 1a. We will test the hypothesis that fear conditioning exerts different effects on PLC- $\beta$ 1a in mice prenatally exposed to alcohol and diet-control mice. Further, we will test the hypothesis that these differences are the result of differences in the effects of fear conditioning on PLC- $\beta$ 1a phosphorylation in these groups of animals. The proposed studies will measure PLC- $\beta$ 1a enzyme activity of dephosphorylated and native enzyme isolated from behavioral control and fear-conditioned mice offspring of dams fed an *ad libitum* or ethanol diet. Identification of the neurochemical abnormalities that underlie the cognitive and behavioral deficits associated with prenatal alcohol exposure will facilitate the development of rational and more effective treatment strategies. This research was supported by NIMH-COR Grant MH-19101, by AA12635 (KKC) and AA06548 (DDS), and U.S. Department of Army Award #DAMD17-00-1-0582 (KKC).

presented at the 2002 National Institute of Mental Health  
Career Opportunities in Research Education and Training  
(COR) Colloquium. April 10-14, 2002, Minneapolis, MN.

Please note that the information contained in this abstract is different from that shown on the poster that was presented at the meeting (miniature version of poster can be found on the following page). This meeting was originally scheduled for September 2001. Because of the tragedies that occurred during that month, the meeting was rescheduled for April 2002. The meeting organizers did not solicit revised abstracts. However, we chose to present our newer data on the effects of fetal alcohol exposure on the association of PLC isozymes and ERK2, rather than our older data on the effects of fetal alcohol exposure on PLC- $\beta$ 1a.



# Fetal Alcohol Exposure Disrupts Signal Integration in Rat and Mouse Hippocampal Formation.

W. Savage, C.T. Buckley, A.M. Allan, D.D. Savage and K.K. Caldwell University of New Mexico, Albuquerque, NM, 87131-5223.

# ABSTRACT

Fetal alcohol exposure (FAE) has been shown to impair learning and memory. Learning and memory have been shown to be dependent on proper functioning of extracellularly regulated protein kinase 2 (ERK2). We investigated the effects of one-trial fear conditioning on phospholipase C (PLC) enzyme activity associated with ERK2 in hippocampal formation postnuclear fractions of untreated control animals and animals perinatally exposed to ethanol. Thirty minutes following conditioning, PLC activity was increased in control rat subcellular fractions, but was unchanged in fetal alcohol-exposed (FAE) animals. Similarly, sixty minutes following conditioning, PLC activity was increased in control mice but not prenatal ethanol-exposed mice subcellular fractions. We speculate that alterations in protein-protein interactions contribute to the learning deficits observed in rats and mice exposed prenatally to ethanol.

## INTRODUCTION

Maternal consumption of moderate levels of ethanol causes subtle cognitive and behavioral aberrations in the offspring (1). We hypothesize that defects in the integration of cellular signaling are important components of the neurochemical imbalances that underlie these cognitive and behavioral abnormalities. Recently, we found that rats who were exposed prenatally to ethanol display reduced retention of learned fear after one trial fear conditioning (2). In the proposed studies, we tested the hypothesis that the integration of hippocampal formation phosphatidylinositol-specific phospholipase C (PLC $\beta$ ) and extracellular signal-regulated kinase (ERK)-dependent signaling is perturbed in adult offspring who were exposed to ethanol prenatally. As a result, behavioral challenge elicits spatially and temporally inappropriate PLC- and ERK-dependent signaling, leading to cognitive and behavioral impairments.

## MATERIALS AND METHODS

*Rats Alcohol Exposure Perinatal*

All of the procedures involving animals were approved by the University of New Mexico Laboratory Animal Care and Use Committee. Pregnant female Sprague-Dawley rats (Taconic, Mexico) were used to develop ethanol drinking in B6SUGL female mice. The mating method was used to develop ethanol drinking in B6SUGL female mice. Mating was initiated on day 1 of estrus. Males and females were housed in pairs in cages with wood chip bedding. The mice were fed a 0.065% saccharin containing 10% ethanol. Blood levels of 120 mg% were achieved. After two weeks of drinking, a male was introduced into the female's cage. One day later, the female was placed in a cage with a male from the F<sub>1</sub> generation to avoid inbreeding depression. Within one day of birth, the ethanol concentration of the maternal milk was determined as follows: day 1, 5.4%; day 3, 2.9%; day 5, 2.1%; and day 7, 0%. Litter sizes and litter weights were the same in the ethanol and control groups. Pups were weighed and weighed again at 18 and 30 days of age. All pups were weaned and weighed again on day 90 days.

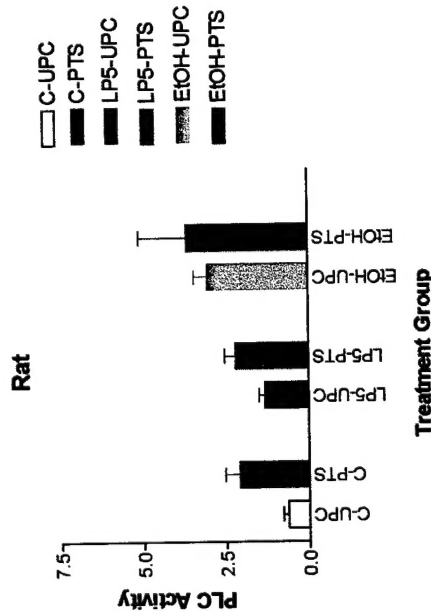
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**Tissue Preparation and Subcellular Fractionation Procedures**

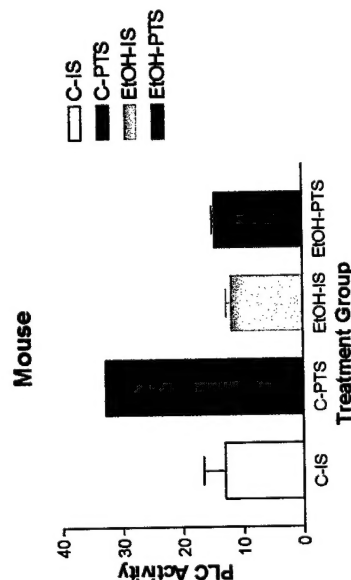
Rats were sacrificed 30 minutes following delivery of the shock, while mice were sacrificed 60 minutes following shock delivery. Both were sacrificed by decapitation. Brains were rapidly removed and the hippocampal formation was dissected. Tissues were homogenized in 10 volumes of 0.25 M sucrose, 0.05 M Tris, and 0.01 M EDTA, pH 7.4. The subcellular fractionation was identical as described (2).

**Immunoprecipitation of ERK2**  
ERK2 was immunoprecipitated from the postnuclear subcellular fraction as described for the immunoprecipitation of PLC- $\beta$ 1s (2).

PLC Enzyme Activity Assay



**Figure 1. PIC activity present in anti-ERK2 immunoprecipitates isolated from rat hippocampal formation postnuclear fractions.** The hippocampal formation was isolated from adult rats that had been prenatally exposed to ethanol (5% ethanol diet, EtOH), or were the offspring of dams receiving an ethanol-free liquid diet (Pair-fed, LPF) or had ad lib access to rat chow (control, C). Following fear conditioning (paired tone-shock, PTD) or behavioral control (unpaired control, UPC), the hippocampal formation was isolated. Postnuclear fractions were prepared and ERK2 immunoprecipitates were isolated. Immunocomplex PIC activity was measured and expressed as pmole [ $^{32}$ P]-ATP/min/mg tissue. Two-way ANOVA revealed a significant effect of diet [ $F(2,21) = 3.35$ ,  $p < 0.02$ ], but no significant effect of fear conditioning, nor a significant interaction between diet and fear conditioning.



**Figure 2** PIC activity present in anti-ERK2 immunoprecipitates isolated from mouse hippocampal formation postnuclear fractions. The hippocampal formation was isolated from adult mice that were prenatally exposed to ethanol (EtOH), or not (control, C), following fear conditioning (paired tone-shock, PIS) or behavioral control (immediate shock, IS). Postnuclear fractions were immunoprecipitated (immunoconcooled). Immunoconcooled fractions were prepared and ERK2 was immunoprecipitated. Immunoconcooled fractions were measured and expressed as nmole  $\text{[}^{32}\text{P]ATP} \cdot \text{min}^{-1} \cdot \mu\text{g tissue}^{-1}$ . Two-way ANOVA revealed a significant effect of diet [ $F(1,6)=13.3$ ,  $p<01$ ], a significant effect of fear conditioning [ $F(1,6)=17.7$ ,  $p<01$ ], and a significant interaction between diet and fear conditioning [ $F(1,6)=10.4$ ,  $p<01$ ].

In these studies we found that the amount of PLC enzyme activity associated with ERK2 in the basal (i.e., UPC) state in fetal alcohol-exposed rat hippocampal formation is significantly higher than in diet-exposed rat hippocampal formation (table 1). This increase in PLC activity in control rats. Further, we did not observe a fear conditioning-dependent increase in the amount of PLC activity associated with anti-ERK2 immunocomplexes in fetal alcohol rats, as was observed in diet control rats. Similarly, in mice exposed to alcohol prenatally, fear conditioning did not increase PLC activity associated with anti-ERK2 immunoprecipitates. However, prenatal ethanol exposure did not increase basal PLC enzyme activity in mice, as was observed in rat hippocampal formation.

## CONCLUSIONS

Recently, it has become appreciated that the components of signal transduction systems often are not isolated from each other, but, rather, are present in the cell as complexes. We have found that one or more PLC isozymes is associated with ERK2 in rat and mouse hippocampal formation. In addition, we found fetal alcohol exposure blocks the effects of fear-conditioned learning and memory on this association. Because ERK2 has been shown to be critical for hippocampal-dependent learning and memory, the impairing effects of fetal alcohol exposure may contribute to the learning deficits observed in animals exposed prenatally to ethanol.

Future studies will aim to identify the PLC isoenzyme(s) that account for the increased basal level of PLC enzyme activity that we have measured in anti-ERK2 immunocomplexes isolated from FAE rat compared to control rats. In addition, studies will aim to uncover the identity of the PLC isoenzymes that account for the increase in anti-ERK2-associated PLC activity following fear conditioning.

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## ACKNOWLEDGMENTS

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A MODERATE EXPOSURE FETAL ALCOHOL MOUSE MODEL  
USING A SACCHARINE FADING TECHNIQUE.

A.M. ALLAN, J. CHYNOWETH AND K.K. CALDWELL

University of New Mexico, Health Sciences Center, Albuquerque, NM  
87131

Previous studies using a rat model of fetal alcohol exposure (FAE), from our laboratory have observed behavioral deficits in Morris water task performance and contextual fear conditioning (CFC). Biochemical changes in phospholipase A2 and Cbeta-1 also have been seen in the rat model. More recently changes in MAP kinase activity, which has been shown to play a role in CFC have also been noted in the rat FAE model. However, concerns about cross-fostering, liquid diet and pair-fed control changes have been raised. Our goal was to develop a moderate fetal alcohol exposure model using the mouse where we could eliminate the issues of diet and need for cross fostering of pups. Our model used a modification of the sucrose fading techniques, developed by Samson and Hodge, to result in high stable voluntary alcohol consumption by the females. Through out the study the mice had ad lib water and breeder chow available. Immediately following delivery the dams were weaned off the alcohol saccharine solution. Pups remained with the moms. Chow intake did not differ between the control and ethanol moms. Litter size and pup weight also did not differ. Average blood alcohol level of the moms was 120 mg%. A deficit in context freezing in the FAE mice was seen. Further, in the saccharin control mice, CFC produced a significant increase in the amount of PLC activity present in anti-ERK2 immunocomplexes compared to those isolated from immediate shock control mice. In contrast, anti-ERK2 immunocomplexes isolated from the FAE mice do not show changes in associated PLC activity following CFC. The saccharin fading technique supports a moderate alcohol consumption in females mice through out pregnancy. The early data using this model suggests similar behavior and neurochemical changes as seen in the rat model, suggesting the changes reported in the rat were due to alcohol exposure and not the result of diet or separation stress. This research was supported by National Institute on Alcohol Abuse and Alcoholism Grants AA12635 (KKC) and U.S. Department of Army Award #DAMD002042 (AMA) and #DAMD17-00-1-0582(KKC).